Identification and characterization of a lysophosphatidylcholine acyltransferase in alveolar type II cells

Xueni Chen*, Brian A. Hyatt†, Michael L. Mucenski‡, Robert J. Mason*§, and John M. Shannon‡

*Department of Medicine, National Jewish Medical and Research Center, 1400 Jackson Street, Denver, CO 80206; †Department of Biology, Bethel University, 3900 Bethel Drive, St. Paul, MN 55112; and ‡Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229-3039

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Pulmonary surfactant is a complex of lipids and proteins produced and secreted by alveolar type II cells that provides the low surface tension at the air–liquid interface. The phospholipid most responsible for providing the low surface tension in the lung is dipalmitoylphosphatidylcholine. Dipalmitoylphosphatidylcholine is synthesized in large part by phosphatidylcholine (PC) remodeling, and a lysophosphatidylcholine (lysoPC) acyltransferase is thought to play a critical role in its synthesis. However, this acyltransferase has not yet been identified. We have cloned full-length rat and mouse cDNAs coding for a lysoPC acyltransferase (LPCAT). LPCAT encodes a 535-aa protein of ≈59 kDa that contains a transmembrane domain and a putative acyltransferase domain. When transfected into COS-7 cells and HEK293 cells, LPCAT significantly increased lysoPC acyltransferase activity. LPCAT preferred lysoPC as a substrate over lysoPA, lysoPI, lysoPS, lysoPE, or lysoPG and prefers palmitoyl-CoA to oleoyl-CoA as the acyl donor. This LPCAT was preferentially expressed in the lung, specifically within alveolar type II cells. Expression in the fetal lung and in rat type II cells correlated with the expression of the surfactant proteins. LPCAT expression in fetal lung explants was sensitive to dexamethasone and FGFs. KGF was a potent stimulator of LPCAT expression in cultured adult type II cells. We hypothesize that LPCAT plays a critical role in regulating surfactant phospholipid biosynthesis and suggest that understanding the regulation of LPCAT will offer important insight into surfactant phospholipid biosynthesis.

biochemistry | lung | surfactant | phosphatidylcholine

Pulmonary surfactant lines the interior of the lungs, and its presence at the air–liquid interface decreases surface tension and thereby prevents alveolar collapse, small airway closure, and alveolar flooding (1). Pulmonary surfactant deficiency is recognized to be an important contributing factor in the pathogenesis of neonatal respiratory distress syndrome (NRDS), acute respiratory distress syndrome (ARDS), and diseases of small airways such as asthma and bronchiolitis. Surfactant is composed predominantly of phospholipids and also contains the surfactant proteins SP-A, SP-B, and SP-C. Dipalmitoylphosphatidylcholine (DPPC) is the phospholipid most responsible for the surface tension-lowering properties of surfactant. Synthesis of DPPC occurs by direct *de novo* synthesis or by remodeling of unsaturated phosphatidylcholine (PC). The direct biosynthesis pathway of DPPC occurs by means of conversion of phosphatidic acid to 1,2-dipalmitoyl diacylglycerol by phosphatidate phosphatase. 1,2-dipalmitoyl diacylglycerol is then converted to DPPC by cholinephosphotransferase. Formation of DPPC by the remodeling mechanism involves deacylation of unsaturated PC at the sn-2 position by a phospholipase A2, followed by reacylation of the resultant 1-palmitoyl-2-lysophosphatidylcholine with palmitoyl-CoA via a lysophosphatidylcholine (lysoPC) acyltransferase. Because of the preferential incorporation of radiolabeled palmitate into the sn-2 position of surfactant DPPC by type II cells and lung microsomes, PC remodeling has been recognized as a major pathway for DPPC synthesis for many years (1–3). It is estimated that 55–75% of the DPPC found in type II cells is formed by this remodeling pathway (4, 5). Although the pathways for phospholipid synthesis in type II cells have been previously defined through metabolic labeling experiments, very little is known about the regulation of these pathways (1, 5, 6). Understanding the regulation of lipid synthesis is potentially important for the development of new therapeutic agents to increase endogenous surfactant.

Only a few glycerol lipid acyltransferases have been cloned and sequenced (7–11). Structural analysis of several glycerolipid acyltransferases from a variety of organisms has revealed a critical domain responsible for their catalytic activity (12). The critical amino acid motif of $H(X)_4D$ is present within this domain in all glycerolipid acyltransferases that have been studied to date (8–11). To our knowledge, the cloning of any lysoPC acyltransferase has not been reported. Although there are undoubtedly several lysoPC acyltransferases with different acyl-CoA preferences, the one involved in the remodeling pathway of DPPC synthesis in type II cells seems to prefer palmitoyl-CoA to oleoyl-CoA as an acyl donor (1).

In a microarray analysis comparing embryonic day (E) E13.5 and E16.5 mouse lungs, we identified an EST that was upregulated 5-fold in the E16.5 lung. In the present study, we have cloned cDNAs of this gene [hereafter referred to as lysoPC acyltransferase (LPCAT)] from both the mouse and rat. Importantly, we demonstrate that rat LPCAT is an acyltransferase that prefers lysoPC as the lysophospholipid substrate and palmitoyl-CoA as the acyl donor. We have determined the temporal and spatial expression of LPCAT in the developing lung and provide information on its regulation *in vitro*. The specificity and the expression pattern of this enzyme indicate that it is likely to be critical in the synthesis of pulmonary surfactant DPPC via the remodeling pathway.

Results

Cloning of Full-Length Mouse and Rat LPCAT cDNAs. In experiments designed to identify genes differentially expressed during lung development, we identified an EST, IMAGE clone 422946, which was expressed 4.9-fold higher in E16.5 versus E13.5 murine lung. We obtained and sequenced the 1.3-kb cDNA for 422946 and found that it did not contain an ORF. However, BLAST analysis against the public mouse database revealed overlapping ESTs that extended the 5′ sequence to give a "virtual clone" of 3.4 kb. RT-PCR performed using primers flanking the putative coding region of the virtual clone and adult

Conflict of interest statement: No conflicts declared.

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; lysoPC, lysophosphatidylcholine; E*n*, embryonic day *n*; LPCAT, lysoPC acyltransferase; ISH, *in situ* hybridization; Dex, dexamethasone.

§To whom correspondence should be addressed. E-mail: masonb@njc.org.

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1   MRLRGRGPRAAPSSSSGAGDARRLAPPGRNPFVHELRLSALQKAQVAFMT
51  LTLFPIRLLFAAFMMLLAWPFALVASLGPPDKEPEQPLALWRKVVDFLLK
101 AIMRTMWFAGGFHRVAVKGRQALPTEAAILTLAPHSSYFDAIPVTMTMSS
151 IVMKAESRDIPIWGTLIRYIRPVFVSRSDQDSRRKTVEEIKRRAQSNGKW
201 PQIMIFPEGTCTNRTCLITFKPGAFIPGVPVQPVVLRYPNKLDTITWTWQ
251 GPGALKILWLTLCQFQNQVEIEFLPVYCPSEEEKRNPALYASNVRRVMAK
301 ALGVSVTDYTFEDCQLALAEGQLRLPADTCLLEFARLVRGLGLKPENLEK
351 DLDKYSESARMKRGEKIRLPEFAAYLEVPVSDALEDMFSLFDESGGGEID
401 LREYVVALSVVCRPSQTLATIQLAFKMYGSPEDGSIDEADLSCILKTALG
451 ISELTVTDLFQAIDQEERGRITFDDFCGFAEMYPDFAEDYLYPDQTHSDS
501 CAQTPPAPTPNGFCIDFSPEHSDFGRKNSCKKVDN
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Fig. 1. LPCAT protein sequence. The structure of LPCAT has a transmembrane domain, a domain for glycerolipid acyltransferase activity, and two EF hand (EFH) domains for potential calcium binding sites. The domain for glycerolipid acyltransferase activity is highlighted, and the critical $H(X)_4D$ signature is underlined.

lung cDNA as a template gave a product of the predicted size of 1.6 kb. This product was cloned and sequenced, and this transcript encodes a putative protein of 534 aa [National Center for Biotechnology Information (NCBI) accession no. NP_663351].

The rat LPCAT cDNA (NCBI accession no. XM_341747) was identified in the NCBI database based on its sequence homology to the mouse LPCAT sequence. We designed primer pairs flanking the coding region of the rat LPCAT and amplified it from alveolar type II cell cDNA. The resultant product, which was 1.6 kb in size, was cloned and sequenced to confirm its identity. The rat LPCAT cDNA translates to a 535-aa protein of ≈59 kDa.

Both mouse and rat LPCAT contain a putative acyltransferase activity domain (Fig. 1). This domain contains conserved His and Asp residues in an $H(X)_4D$ configuration, which is necessary for acyltransferase activity (12). Alignment of the amino acid sequences of mouse and rat LPCAT (NCBI accession nos. NP_663351 and XP_341748, respectively) showed that they are 99% identical. We also identified the human ortholog of LPCAT (NCBI accession no. NP_079106) and determined that it is 89% homologous with the mouse and rat sequences.

Acyltransferase Activity of LPCAT. We used a mammalian expression system to achieve high-level expression of rat LPCAT in HEK293 cells and COS-7 cells for the functional characterization of the enzyme. To facilitate Western blot analysis, we attached a V5 tag to the C terminus of LPCAT. Western blots of HEK293 cells and COS-7 cells transfected with LPCAT and probed with anti-V5 antibody gave a band of 63 kDa (Fig. 2*A*). This size is consistent with the molecular weight predicted from the LPCAT ORF. We then determined whether LPCAT had acyltransferase activity. Because PC is the most abundant surfactant phospholipid, we first tested the acyltransferase activity of LPCAT using lysoPC as the acyl-accepting substrate. LysoPC acyltransferase activity was determined by conversion of lysoPC to PC in the presence of [^{14}C]palmitoyl-CoA. We found that lysoPC acyltransferase activity was increased 10-fold in HEK293 cells transfected with LPCAT compared with empty vector controls. We next determined the specificity of LPCAT acyltransferase activity using a variety of lysophospholipids as substrates (Fig. 2*B*). We detected no significant increase in acyltransferase activity when lysoPE, lysoPI, and lysoPS were used as substrates and slight activity with lysoPA and lysoPG as

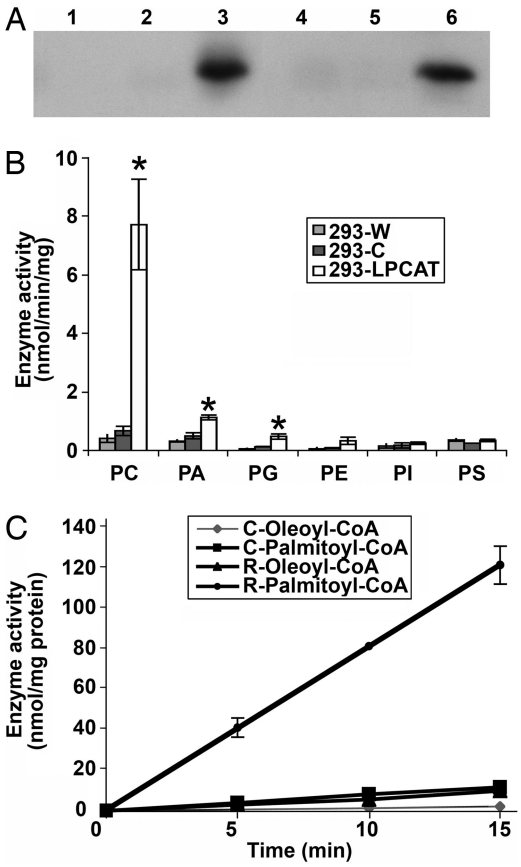

Fig. 2. LysoPC acyltransferase activity. (*A*) Expression of rat LPCAT in mammalian cells. Forty-eight hours after transfection, cells were processed for Western blot using an anti-V5 antibody. Lane 1, wild-type COS-7 cells; lane 2, COS cells transfected with the empty vector; lane 3, COS-7 cells transfected with LPCAT; lane 4, wild-type HEK293 cells; lane 5, HEK293 cells transfected with the empty vector; lane 6, HEK293 cells transfected with LPCAT. (*B*) The acyltransferase activities of the recombinant LPCAT expressed in HEK293 cells with different lysophospholipid substrates. The acyltransferase assays were conducted by incubating 20 μM [1-^{14}C]palmitoyl-CoA with each of the lysophospholipids (150 μM) in the presence of 20 μg of cell homogenates from either wild-type cells (293-W), cells transfected with control vector (293-C), or with LPCAT (293-LPCAT). All enzyme activity data are derived from at least three independent experiments and are shown as mean ± SE. *, a significant difference ($P < 0.001$) from the vector control. (*C*) The preference of palmitoyl-CoA over oleoyl-CoA on lysoPC acyltransferase activity of recombinant (R) LPCAT expressed in HEK293 cells compared with C (control, empty vector). The enzyme activity was performed by incubating 150 μM lysoPC with 20 μM each [^{14}C]acyl-CoA for 0, 5, 10, and 15 min. The data are derived from at least three independent experiments and are shown as mean ± SE.

substrates. These results indicate that LPCAT functions predominantly as a lysoPC acyltransferase.

LPCAT Shows a Preference for Palmitoyl-CoA over Oleoyl-CoA. Having determined that lysoPC is the preferred substrate of LPCAT, we next analyzed its acyl selectivity. We incubated lysates of HEK293 cells expressing LPCAT with lysoPC as the acyl-accepting substrate and [1-^{14}C]palmitoyl-CoA (16:0) and [1-^{14}C]oleoyl-CoA (18:1) as potential acyl donors. We chose these acyl donors for comparison because they show differences in utilization with type II cells compared with whole lung (13). LPCAT demonstrated a clear preference for palmitoyl-CoA over oleoyl-CoA as the acyl donor, which is consistent with the composition of surfactant lipids in alveolar type II cells (Fig. 2*C*).

Effect of Divalent Cations on LPCAT Activity. We also found that the addition of magnesium or manganese stimulated the enzymatic

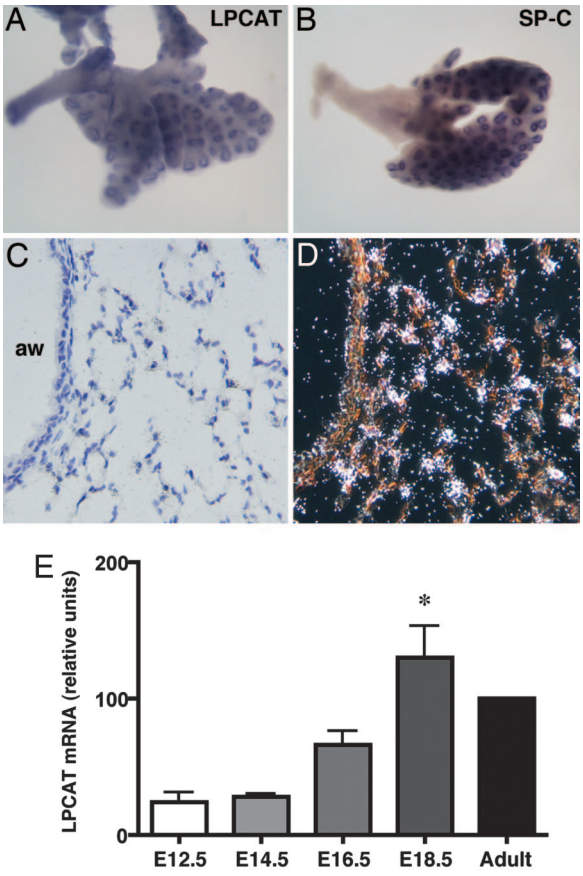

Fig. 3. LPCAT expression in the developing and adult lung. Lungs were harvested from mouse embryos on days E13.5, E15.5, and E18.5, as well as from adults. Whole mount ISH on day E13.5 (*A*) showed that LPCAT was expressed intensely in the distal epithelial tips but not in proximal major airways. This expression pattern was identical to that seen for SP-C (*B*). Lungs hybridized with a sense LPCAT probe gave no signal (data not shown). Sections of E15.5 and E18.5 lung hybridized with LPCAT probe showed specific localization to distal acini, whereas large airways were negative. In the adult lung (*C* and *D*), LPCAT expression was highest in epithelial cells of the alveolar wall and was not present in airway (aw) epithelium. (*E*) Real-time PCR was used to determine LPCAT mRNA expression levels in the developing lung normalized to β-actin and compared with the levels in adult lung. The results are means ± SEM from three independent determinations at each gestational age. *, significantly ($P < 0.01$) increased compared with days E12.5, E14.5, and E16.5.

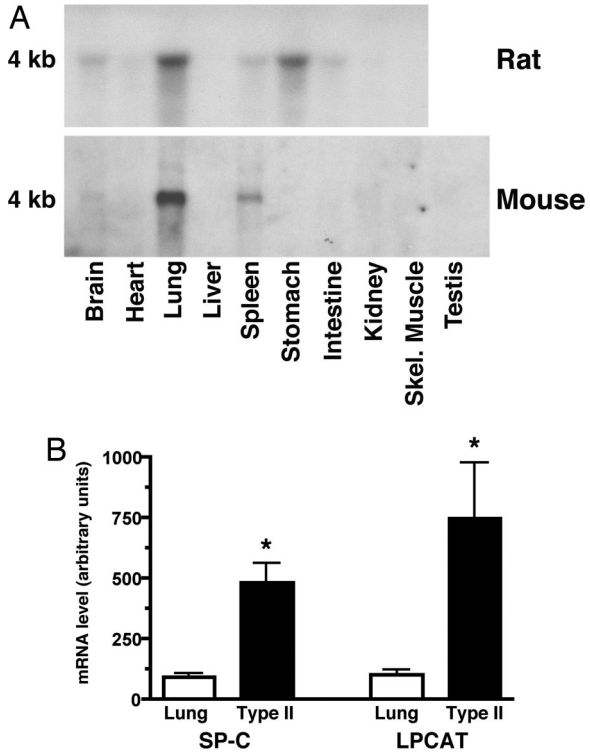

Fig. 4. LPCAT expression in adult organs. (*A*) Northern blots were prepared by using 10 μg of total RNA from various rat and mouse organs, then hybridized with radiolabeled mouse LPCAT cDNA. Expression was most abundant in the lung for both rat and mouse. The rat also showed significant expression in the stomach, along with faint expression in the brain, spleen, and intestine. In the mouse, faint expression was also detected in the spleen. (*B*) Real-time PCR was used to compare LPCAT and SP-C mRNA content in adult mouse lungs and freshly isolated mouse alveolar type II cells. Normalized to β-actin, LPCAT content was enriched 7.4-fold in type II cells, and SP-C content was enriched 4.8-fold. The results are means ± SEM from four independent experiments. *, significantly greater ($P < 0.05$) than whole lung.

activity of LPCAT. Optimal activity was obtained with 5–10 mM Mg^{2+} (data not shown). Manganese also stimulated LPCAT activity, showing an optimal concentration of 2 mM in the absence of Mg^{2+} (data not shown).

Expression of LPCAT in the Developing Lung. Because our original microarray suggested that LPCAT was developmentally regulated, we determined the spatial and temporal expression of LPCAT in the developing mouse lung. We found by whole mount *in situ* hybridization (WM-ISH) that LPCAT was widely expressed in the lung parenchyma on day E13.5 (Fig. 3*A*) but was not present in the major airways. This expression pattern seemed identical to that seen for SP-C (Fig. 3*B*). Tissue section ISH of lungs on days E15.5 and E18.5 showed that LPCAT expression became more restricted to the most distal epithelial cells with increasing gestational age (data not shown). LPCAT expression in the adult mouse lung appeared predominantly in cells along the alveolar wall, presumably alveolar type II cells (Figs. 3 *C* and *D*). We also performed real-time PCR to quantify LPCAT expression during murine lung development. By using the adult level of LPCAT expression normalized to β-actin as 100%, we found that relative LPCAT expression was 23%, 27%, 66%, and 130% on days E12.5, E14.5, E16.5, and E18.5, respectively (Fig. 3*E*).

LPCAT Is Abundantly Expressed in the Lung and Alveolar Type II Cells. If LPCAT is specifically involved in the synthesis of DPPC destined for pulmonary surfactant as opposed to membrane biogenesis, then LPCAT expression should be higher in the lung than in other organs. To examine this hypothesis, we generated Northern blots of various mouse and rat organs probed for LPCAT expression (Fig. 4*A*). We found the expression pattern between the mouse and rat to be very similar. In both species, we found that, in the organs examined, LPCAT was most abundantly expressed in the lung. LPCAT mRNA was faintly expressed in the mouse spleen, but not in brain, heart, liver, stomach, intestine, kidney, skeletal muscle, and testis. In the rat, we observed significant LPCAT expression in the stomach, with faint expression in the spleen, brain, and intestine. To confirm our ISH data indicating that LPCAT is highly expressed in adult alveolar type II cells, we used real-time PCR to compare its relative expression in whole lung versus isolated type II cells. As a basis for comparing the extent of enrichment, we also measured the relative content of SP-C, which is expressed only in type II cells in the lung. We found that LPCAT was enriched 7.4-fold in isolated type II cells compared with whole lung, a level of

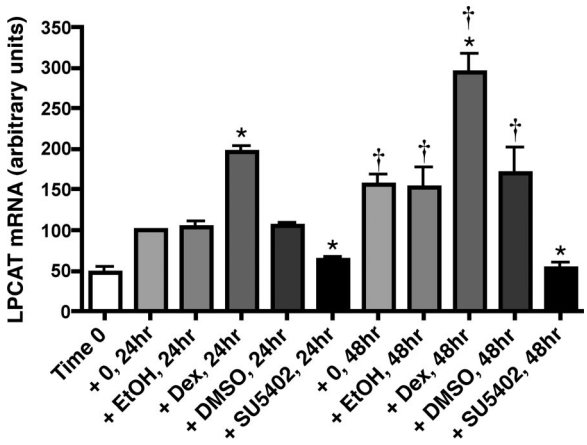

Fig. 5. LPCAT expression is regulated by glucocorticoids and FGFs in the developing lung. E16.5 lung explants were cultured for 1–2 days in medium containing 10^{-7} M Dex or 10 μM SU5402; 0.1% ethanol (EtOH) or 0.1% DMSO served as respective vehicle controls. LPCAT mRNA content, normalized to β-actin, was determined by real-time PCR. The results are means ± SE from three independent experiments. Compared with time 0 tissue, LPCAT expression was significantly increased ($P < 0.001$) in the presence of EtOH, DMSO, or no additions (+0) at both 24 and 48 h of culture. Compared with EtOH, the addition of Dex caused a significant ($P < 0.001$) increase in LPCAT expression at both 24 and 48 h. In contrast, abrogation of FGF signaling with SU5402 significantly ($P < 0.01$) inhibited the increase in LPCAT expression seen in DMSO treated cultures. *, significantly different from vehicle control; †, significantly different from the same treatment at 24 h.

enrichment that was similar to that observed for SP-C (4.8-fold) (Fig. 4*B*).

Glucocorticoids and FGFs Affect LPCAT Expression in the Developing Lung. Prenatal lungs exposed to glucocorticoids show an increase in the rate of DPPC synthesis. Because our data indicate that LPCAT functions in DPPC synthesis, we hypothesized that one way in which glucocorticoids could affect DPPC synthesis is by increasing expression of LPCAT. To test this theory, we cultured mouse lung explants for 1–2 days in the presence of 10^{-7} M dexamethasone (Dex). KGF (FGF7) has been also been shown to enhance DPPC synthesis in the late gestational lung (14). To determine whether LPCAT expression was downstream of FGF signaling, we incubated E16.5 explants with the FGF receptor antagonist SU5402. We found that LPCAT expression increased with time in culture in the absence of any treatment (Fig. 5). Dex treatment also significantly increased LPCAT expression, whereas abrogating FGF signaling with SU5402 prevented any increase in LPCAT expression.

KGF Increases LPCAT Expression in Cultured Adult Rat Type II Cells. If LPCAT were involved with surfactant synthesis in adult type II cells, one would expect it to be increased by KGF and to be decreased when the cells are cultured on plastic (15). KGF increased LPCAT mRNA 8-fold in type II cells cultured on a matrix permissive of differentiation (Fig. 6*A*). In addition, when type II cells are cultured on tissue culture plastic, they rapidly lose markers of the type II cell phenotype (e.g., surfactant proteins and DPPC synthesis) while concomitantly increasing expression of some type I cell markers. If LPCAT is critical for surfactant synthesis and is not related to general membrane biogenesis, we would expect it to decrease in type II cells when they dedifferentiate on plastic. As shown in Fig. 6, when rat type II cells are cultured on plastic for 2 and 4 days, the expression of LPCAT and SP-A (a type II cell marker) decreased, whereas the expression of T1α increased (16). These results strongly suggest that LPCAT is involved with surfactant production and not general membrane biogenesis.

Discussion

Although the pathways for surfactant phospholipid synthesis have been defined by metabolic labeling experiments, little is known about the regulation of the enzymes involved in individual steps (1). Regulation of these enzymes in type II cells is crucial for production of surfactant phospholipids. The most abundant surfactant phospholipid is PC. Surfactant PC differs

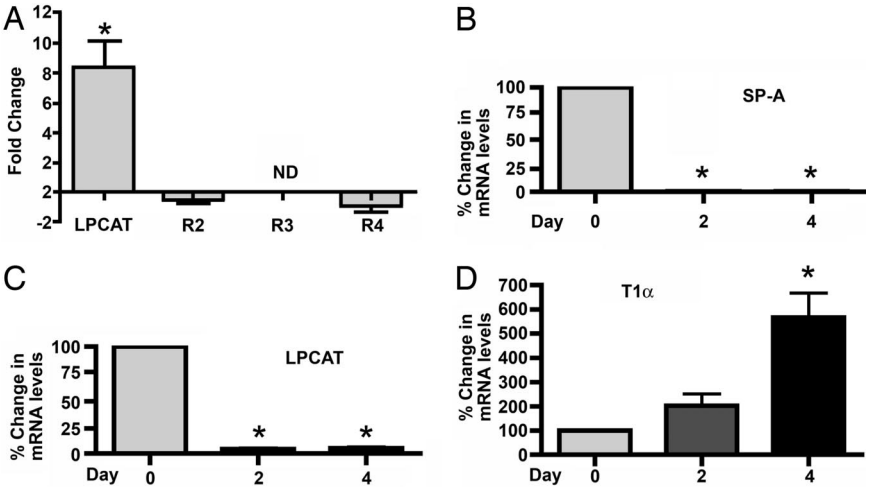

Fig. 6. Regulation of LPCAT in type II cells. (*A*) mRNA levels of LPCAT are increased by KGF in rat alveolar type II cells. Type II cells were cultured for 5 days with and without KGF (10 ng/ml). The mRNA levels were determined by real-time PCR, as described in *Materials and Methods*. The results were normalized with cyclophilin B and expressed as a fold change in comparison with the cultures without KGF. The results are the means ± SE for five experiments. *, a significant increase ($P < 0.01$) from the control culture. KGF increased LPCAT levels (R1), whereas the expression of the other putative acyltransferases (R2–R4) was unchanged. The mRNA for R3 was below the level of detection (ND). (*B–D*) Rat alveolar type II cells were cultured in plastic wells with 10% FBS. Cells were harvested on the indicated days. The mRNA levels of LPCAT, SP-A (type II cell marker), and T1α (type I cell marker) were examined by real-time PCR and normalized to GAPDH. (*B*) The mRNA expression of SP-A decreased during the *in vitro* transition from type II cells to type I-like cells. (*C*) The expression of LPCAT decreased in parallel with SP-A, whereas the expression of T1α increased as expected under these culture conditions (*D*). The results are the means ± SE for three experiments. *, a significant difference ($P < 0.05$) from the control culture.

from most other tissue PCs by its high concentration of 16:0/16:0 PC (DPPC) (17). DPPC is synthesized by *de novo* and remodeling pathways. The remodeling pathway, which involves deacylation of PC followed by reacylation of lysoPC with palmitoyl-CoA, is thought to account for 55–75% of DPPC synthesis (4, 5). LysoPC acyltransferase, which catalyzes this reacylation, therefore likely plays an important role in DPPC synthesis. Previous biochemical studies using lung and type II cell microsomes demonstrated a lysoPC acyltransferase (EC 2.3.1.23) that is enriched in type II cells (13, 18, 19). This enzyme also has a higher activity toward palmitoyl-CoA than oleoyl-CoA as does the LPCAT described in this report (13, 20, 21). Similar to the tissue distribution of DPPC, we found that LPCAT was abundantly expressed in the lung compared with other organs. One other organ that seemed to have significant LPCAT expression was the rat stomach. Interestingly, a small amount of DPPC is secreted in the rat stomach, where it has been suggested to provide a mucosal protective barrier (22). LPCAT expression was also enriched in alveolar type II cells in the lung.

The LPCAT gene that we have identified encodes an enzyme that catalyzes the acylation of lysoPC. Notably, this acyltransferase activity is relatively specific for lysoPC, as we observed only minimal increases in acyltransferase activity with other lysophospholipid substrates. Taken together, our data indicate that we have cloned the cDNA for the type II cell lysoPC acyltransferase that has been characterized in earlier reports. Although a variety of other 1-acyl-lysoglycerolipid acyltransferases have been cloned, none have been shown to be a lysoPC acyltransferase.

The production of surfactant in the fetal lung increases sharply toward the end of gestation (23). Glucocorticoids have been extensively studied for their ability to accelerate fetal lung maturation and stimulate surfactant production. One mechanism by which total DPPC content may be augmented by glucocorticoids is by increased expression of LPCAT. The mRNA levels of LPCAT increased significantly toward the end of gestation. We also found that LPCAT expression was increased by Dex *in vitro*. This observation is in agreement with the results of Oldenborg and Van Golde (24), who observed that maternally administered Dex caused a doubling of lysoPC acyltransferase activity in the lungs of late-gestation murine fetuses. In addition to the positive effects of Dex on LPCAT expression, we found that treating fetal lung explants with SU5402, which blocks signaling through FGF receptors, totally abrogated the endogenous increase in LPCAT expression. FGF family members play important roles in several aspects of lung growth and differentiation (25). Therefore, in the fetal lung, LPCAT is regulated like other components of the surfactant system, which supports the concept that this enzyme is important for DPPC synthesis in surfactant.

The expression of LPCAT is also similar to expression of other components of the surfactant systems in adult type II cells. In adult rat type II cells, KGF greatly increases lipogenesis and the synthesis of disaturated PC (26). KGF also increases the expression of fatty acid synthase, stearoylCoA desaturase 1, and epidermal fatty acid binding protein (15). SREBP-1c, C/EBPα, and C/EBPδ have been suggested to be components of the complex pathway whereby KGF stimulates lipogenesis in type II cells. We found that KGF increased LPCAT expression in adult type II cells but had no effect on three other putative acyltransferases (XM_215783, XM_223143, and XM_226357), indicating that LPCAT is specifically regulated by KGF. LPCAT expression dramatically decreased when type II cells cultured on tissue culture plastic dedifferentiated to a type I-like cell phenotype. These data suggest that LPCAT is increased with type II cell differentiation and surfactant production and does not function in general membrane biogenesis.

In summary, we have identified a lysoPC acyltransferase, LPCAT, that seems to catalyze the conversion of lysoPC to DPPC in alveolar type II cells. The identification of LPCAT has significant implications for understanding the regulation of surfactant phospholipid biosynthesis and may lead to new strategies for prevention and treatment of lung diseases resulting from surfactant deficiency. In addition, identification of LPCAT as a lysoPC acyltransferase may facilitate discovery of other lysoPC acyltransferases.

Materials and Methods

Alveolar Type II Cell Isolation and Culture. Rat alveolar type II cells were isolated and cultured as described (15, 27). A type I-like cell phenotype was generated by culturing type II cells on plastic dishes in DMEM plus 10% FBS without KGF (16). Freshly isolated mouse alveolar type II cells were isolated from adult C57BL/6 mice as described (28).

Fetal Lung Explant Culture. Lungs were dissected from day E16.5 FVB/N mouse fetuses (Taconic, Germantown, NY) and cultured on 8-μm pore size Nucleopore filters in DMEM/F12 medium plus 5% FBS. Explants were treated with 10^{-7} M Dex or with 10 μM SU5402 (Calbiochem, La Jolla, CA) to inhibit signaling through FGF receptors. Ethanol (0.1%) and DMSO (0.1%) (Sigma) served as vehicle controls for Dex and SU5402, respectively.

Isolation of RNA. Type II cells and fetal lung explants in the apical culture system were directly lysed into 4 M guanidinium isothiocyanate, 0.5% *N*-laurylsarcosine, and 0.1 M 2-mercaptoethanol in 25 mM sodium citrate buffer (GITC) and isolated as described (29).

RT-PCR and Real-Time PCR. A rat cDNA clone (NCBI accession no. XM_341747) was identified in NCBI databases based on sequence homology to the mouse putative acyltransferase sequence. According to the flanking cDNA ends, primer pairs (forward, 5′-GCCATGAGGCTGCGGGGTCGCGGGC-3′ and reverse, 5′-GTCCACTTTCTTACACGAATTCTTTC-TCCCAAAGTC-3′) were designed to amplify the full-length coding region of the rat gene from rat alveolar type II cell cDNA. The resultant PCR product was 1.6-kb and was cloned into pET101/D-TOPO vector (Invitrogen, Carlsbad, CA), and the sequence was verified. Primers and probes for real-time PCR of rLPCAT were designed by using Primer Express 1.5a software (Applied Biosystems, Branchburg, NJ). The primers and probes for SP-A and T1α have been reported (15, 30). The forward primer for rLPCAT was 5′-CTCCTGAGGATG-GCAGCATAG-3′, the reverse primer was 5′-TGATATGC-CCAGTGCAGTCTTG-3′, and the probe was 5′-TGAAGC-CGACCTGTCCTGCATCC-3′. Rat cyclophilin B or GAPDH was used to normalize LPCAT expression in all assays. The reactions were quantified by selection of the amplification cycle during which the PCR product of interest was detected to be accumulating logarithmically [the threshold cycle (C_T) method] as reported (15).

cDNA synthesized from embryonic mouse lung tissues (Improm-II reverse transcription, Promega, Madison, WI) was subjected to real-time PCR analysis by using SYBR Green and a Smart Cycler (Cepheid, Sunnyvale, CA) as described (31). LPCAT mRNA expression was normalized to β-actin. The mouse LPCAT forward primer was 5′-GGCTCCACATTCCTC-CTACTTTG-3′ and the reverse primer was 5′-ATCTCCTC-CACTGTCTTCCTTCG-3′. The β-actin primers were as described (31). Relative quantitation was obtained by measuring the cycle at which the greatest accumulation of product occurred (cycle threshold) and plotting that against the cycle thresholds of a dilution series of positive control samples.

Northern Blot Analysis. Total RNA from different rat and mouse organs was purified by CsCl ultracentrifugation as described above. Northern blot analysis was performed as described (29).

***In Situ* Hybridization.** Tissues for whole mount ISH (WM-ISH) were fixed in freshly prepared 4% paraformaldehyde in PBS. WM-ISH was performed by using digoxigenin-labeled antisense or sense probes transcribed from full-length cDNAs for mouse LPCAT and SP-C by using a Superscript kit (Promega). Tissue section ISH was performed as described (32).

Expression of LPCAT in Mammalian Cells. The entire coding region of rat LPCAT was amplified by PCR and subcloned from pET101/D-TOPO into the expression vector pIRES-EYFP (BD Biosciences Clontech, Palo Alto, CA). The amplified DNA fragment was cloned into the pIRES vector, designated as prLPCAT, and verified by sequencing. Transient transfection was performed by using COS-7 and HEK293 cells. Twenty-four hours before transfection, 2×10^6 cells were plated into 20 × 100-mm plates, which resulted in 60–70% confluence at the time of transfection. Cells were transfected with 8 μg of DNA by using PolyFect (Qiagen, Valencia, CA) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested, washed with PBS, and disrupted with a probe sonicator in homogenization buffer [10 mM Tris·HCl/1 mM EDTA, pH 7.4/250 mM sucrose/1× protease inhibitor mixture (BD Biosciences PharMingen, San Jose, CA)]. The homogenate was aliquotted and stored at −70°C. Transfection was monitored by Western blot analyses for the V5 epitope.

***In Vitro* Acyltransferase Activity Assays.** Acyltransferase activity was determined by measuring the incorporation of radiolabeled acyl-CoAs (acyl donors) into phospholipids in the presence of relevant lysophospholipids (acyl acceptors). The lysophopholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Enzyme activity was determined by conversion of lysophospholipid to phospholipid in the presence of acyl-CoA. The reaction mixture (200 μl final volume) contained 80 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 200 μM each lysophospholipid, 25 μM [1-^{14}C]acyl-CoA (PerkinElmer Life Sciences, Boston, MA) and 20 μg of lysate from HEK293 cells transfected with LPCAT or empty vector. The reaction was initiated by the addition of the protein homogenate and was terminated by adding 1 ml of chloroform/methanol (2:1, vol/vol). Experiments for determining the substrate preference of LPCAT were terminated after 10 min of incubation at 25°C. For determining the acyl donor preference of LPCAT, 20 μg of lysate from HEK293 cells transfected with LPCAT or empty vector were incubated with 200 μM lysoPC and 20 μM [1-^{14}C]palmitoyl-CoA or [1-^{14}C]oleoyl-CoA (PerkinElmer Life Sciences) for 0, 5, 10, and 15 min.

The phospholipids were extracted by the method of Bligh and Dyer (33). Aliquots of the organic phase containing phospholipids were dried and separated by TLC, by using chloroform/methanol/water/acetic acid (140/60/8/4) as the solvent system for PC, phosphatidylglycerol, and phosphatidylethanolamine (Silica Gel G plate, Analtech, Inc., Newark, NJ), and chloroform/acetic acid/methanol/water (108/36/7.2/3.1) for phosphatidylinositol, phosphatidylserine, and phosphatidic acid (Silica Gel G60, EMD Chemicals, Darmstadt, Germany). Identities of the different phospholipids were verified with lipid standards after staining with iodine vapor. Individual phospholipid species were transferred to scintillation vials, and radioactivity was quantified by liquid scintillation counting.

Statistics. Results for real-time PCR and for enzymatic activity were subjected to one-way ANOVA by using a Newman–Keuls multiple comparison test.

Note Added in Proof. After the submission of this manuscript, a paper by Nakanishi *et al.* describing the cloning and characterization of mouse LPCAT has appeared as an electronic publication (34).

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